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Colonization of Mucin by Human Intestinal Bacteria and Establishment of Biofilm Communities in a Two-Stage Continuous Culture System

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The human large intestine is covered with a protective mucus coating, which is heavily colonized by complex bacterial populations that are distinct from those in the gut lumen. Little is known of the composition and metabolic activities of these biofilms, although they are likely to play an important role in mucus breakdown. The aims of this study were to determine how intestinal bacteria colonize mucus and to study physiologic and enzymatic factors involved in the destruction of this glycoprotein. Colonization of mucin gels by fecal bacteria was studied in vitro, using a two-stage continuous culture system, simulating conditions of nutrient availability and limitation characteristic of the proximal (vessel 1) and distal (vessel 2) colon. The establishment of bacterial communities in mucin gels was investigated by selective culture methods, scanning electron microscopy, and confocal laser scanning microscopy, in association with fluorescently labeled 16S rRNA oligonucleotide probes. Gel samples were also taken for analysis of mucin-degrading enzymes and measurements of residual mucin sugars. Mucin gels were rapidly colonized by heterogeneous bacterial populations, especially members of the *Bacteroides fragilis* group, enterobacteria, and clostridia. Intestinal bacterial populations growing on mucin surfaces were shown to be phylogenetically and metabolically distinct from their planktonic counterparts.

Epithelial surfaces in the human gastrointestinal tract are covered by a layer of mucus, which prevents most microorganisms reaching and persisting on the mucosal surface (13). This viscoelastic gel (1) is protective against adhesion and invasion by many pathogenic microorganisms, bacterial toxins, end products of metabolism, pancreatic endopeptidases, microbial antigens, and other damaging agents present in the lumen of the bowel. The mucus gel mainly consists of water (ca. 95%) and glycoproteins (1 to 10%), which are responsible for its viscosity and gel-forming properties (18), as well as electrolytes, proteins, antibodies, and nucleic acids (1).

Mucins are chemically and structurally diverse molecules, but they invariably contain large quantities of galactose and hexosamines with lesser amounts of fucose. Strongly polar groups such as neuraminic (sialic) acids and sulfate are also present, although to a highly variable degree (49). The carbohydrate moieties occur as linear and branched oligosaccharides, which can constitute up to 85% of the molecule by weight (53). These oligosaccharides are attached to a protein core via serine or threonine residues. The attachment of sulfate and sialic acids to terminal mucin oligosaccharides confers resistance to digestion by glycosidases (8). Colonic bacteria must therefore be able to synthesize a variety of hydrolytic enzymes (glycosidases, proteases, peptidases, and sulfatases) to completely degrade mucus.

Mucins are likely to be important sources of carbohydrate for saccharolytic bacteria growing in the large intestine, particularly in the distal bowel, where the supply of fermentable carbohydrate is usually limiting (30). As well as being produced by goblet cells in the colonic mucosa, salivary, gastric, biliary, bronchial, and small intestinal mucins also enter the colon in effluent from the small bowel. Particulate matter, such as partly digested plant cell materials, is entrapped in this viscoelastic gel, which must be broken down or circumvented to facilitate access of intestinal microorganisms to the food residues. It is estimated that between 2 to 3 g of mucin enter the large bowel each day from the upper digestive tract (54), but due to experimental difficulties, rates of colonic mucus formation in the healthy human colon are unknown.

Some bacteria can invade the mucus layer, and many intestinal microorganisms use these molecules as carbon, nitrogen, and energy sources (7, 28, 36, 42, 43, 57). The removal of carbohydrates and other components from the glycoprotein, such as sulfate, compromises its protective function in the gut (51, 52), especially when the rate of mucus breakdown exceeds the rate of its synthesis and secretion.

Large complex polymers such as mucin need to be degraded by several different hydrolytic enzymes to smaller oligomers, monosaccharides, and amino acids before they can be assimilated by intestinal microorganisms. Pure and mixed culture studies have established that in many gut bacteria, synthesis of these enzymes, particularly β-galactosidase, *N*-acetyl β-glucosaminidase, and neuraminidase, is catabolite regulated (26, 28, 39) and therefore dependent on local concentrations of mucin and other carbohydrates. While some colonic microorganisms can produce several different glycosidases, which allows them to extensively digest heterogeneous polymers (5, 31, 46), the majority of experimental data point to the fact that the

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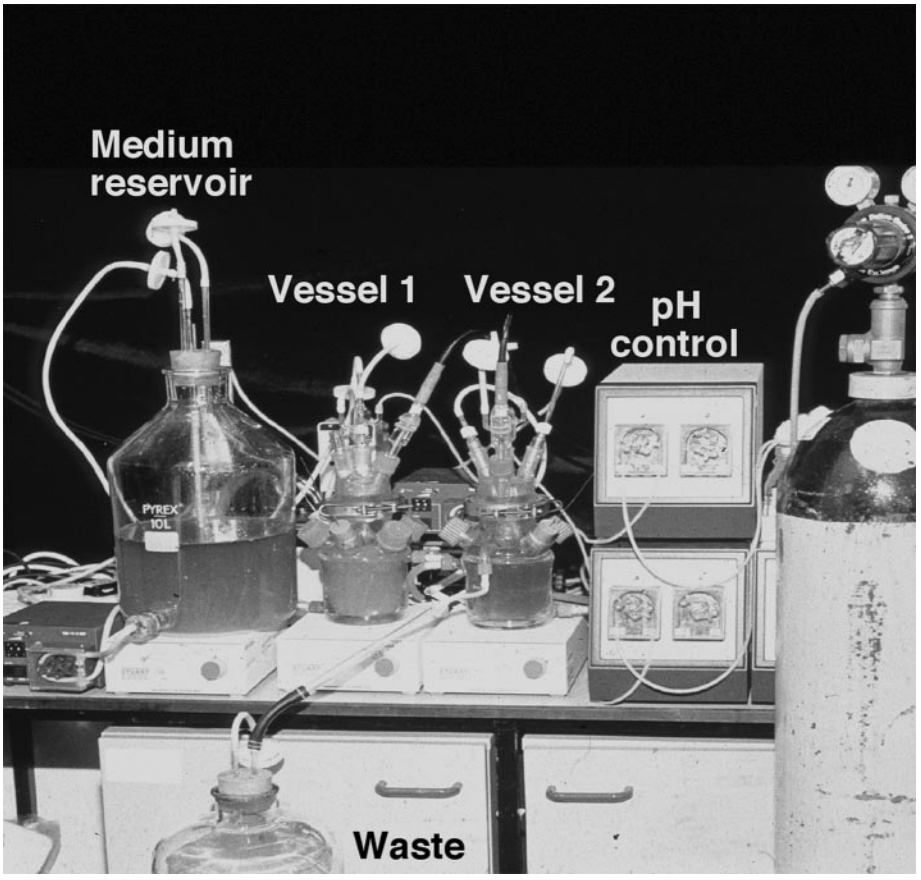

FIG. 1. The two-stage continuous culture system used to simulate bacterial colonization of mucin under nutrient-excess (vessel 1) and nutrient-limited (vessel 2) conditions.

breakdown of mucin and other complex organic molecules is a cooperative activity in the gut microbiota (32, 34).

Mucins and other host-produced glycoproteins such as chondroitin sulfate and hyaluronic acid are able to support the growth of complex bacterial communities in the large intestine in the absence of dietary nutrients (44). Despite its undoubted ecological significance with respect to species composition and the maintenance of stability in the gut ecosystem, few studies have been made of mucosal bacterial communities, due to the physical inaccessibility of the healthy colon for routine studies. There is evidence to suggest that mucosal populations are distinct from those in the lumen (38), and these communities are of increasing interest in that they are thought to play an important role in inflammatory bowel diseases such as ulcerative colitis (35). However, little is known about bacterial growth in the mucus layer or of the organisms involved in its breakdown.

The aims of this work were to investigate colonization of artificial mucus gels by fecal bacteria under conditions of nutrient stress characteristic of the proximal and distal colons, to study community structure and utilization of mucus oligosaccharides during biofilm formation, and to examine physiologic and enzymatic factors that enable the major mucin-degrading species to compete for substrates in biofilms.

MATERIALS AND METHODS

Fermentation system. Two glass fermentation vessels (each, 560-ml working volume) connected in series (Fig. 1) were used in these experiments to simulate conditions of nutrient availability and limitation characteristic of the proximal (vessel 1) and distal (vessel 2) colon. Fresh feces from a healthy volunteer (female, 38 years old) were used to prepare inocula (20% [wt/vol] in 100 mM anaerobic sodium phosphate buffer, pH 6.0). The stools were macerated in a stomacher for 5 min and then sequentially filtered through 500-μm and 250-μm metal sieves to remove large food particles. Two hundred milliliters of this inoculum was added to 200 ml of double-strength culture medium in the fermentors. The vessels were continuously stirred (180 rpm), and each was operated at a dilution rate of 0.10/h. Culture pH was maintained at 6.0 and a temperature of 37°C. Cultures were sparged with O_2-free N_2 gas (2.4 liter/h). Sterile fresh culture medium which was autoclaved, cooled, and maintained under O_2-free N_2 gas was pumped to vessel 1 to create a carbohydrate-rich environment, which subsequently fed vessel 2, which was more carbon depleted. In grams per liter, the growth medium consisted of starch (Lintner's), 2.0; pectin (citrus), 0.5; inulin (chicory), 0.5; xylan (oat spelt), 0.5; arabinogalactan (larchwood), 0.5; guar gum, 0.5; partially purified porcine gastric mucin (grade III; Sigma), 2.0; tryptone, 3.0; peptone water, 3.0; yeast extract, 4.5; hemin, 0.015; NaCl, 4.5; KCl, 2.5; $MgCl_2 \cdot 6H_2O$, 0.45; $CaCl_2 \cdot 6H_2O$, 0.2; KH_2PO_4, 0.4; cysteine, 0.8; and bile salts no. 3, 0.4. In addition, 2 ml of a trace element solution (3) and 0.5 ml of Tween 80 were added per liter of culture medium. When the chemostats reached a metabolic steady state, as determined by monitoring short-chain fatty-acid (SCFA) formation (>10 culture turnovers), samples of the planktonic phase were taken for chemical and enzymatic measurements and for viable counts of bacteria, using a range of selective and nonselective agars. Sterile porcine mucin gels (2% [wt/vol]) in glass tubes (17 by 12 mm; 2-ml volume) containing 0.8% (wt/vol) bacteriological agar (pH 6.0) were then placed in the fermentors and removed

periodically over a period of 48 h for enzymatic, carbohydrate, and bacteriological analyses.

Microbiological studies. Samples from the fermentors and mucin gels were serially diluted in half-strength anaerobic peptone water and plated onto a variety of selective and nonselective agars. Plates used for isolation of facultatively anaerobic organisms were as follows: nutrient agar, total facultative anaerobes; MacConkey agar no. 2, lactose-fermenting and nonfermenting enterobacteria and enterococci; azide blood agar base, facultatively anaerobic cocci. Isolation media for strictly anaerobic bacteria were Wilkins-Chalgren agar (WCA), for total anaerobes, eubacteria, anaerobic gram-positive cocci, and clostridia; WCA with addition of nonsporing anaerobe supplements (to prevent growth of spore-forming species); WCA with gram-negative anaerobe supplements, which was selective for gram-negative organisms; Mann-Rogosa-Sharpe medium, for lactobacilli. Bacteria belonging to the *Bacteroides fragilis* group were isolated and enumerated using *Bacteroides* mineral salts agar (31), bifidobacteria were counted on Beerens agar (4), and fusobacteria were counted on fusobacterium agar (40). Plates for aerobic incubation were removed from the anaerobic chamber and incubated at 37°C. Aerobic plates were incubated for 2 days and anaerobic plates were incubated for up to 5 days, with periodic examination before the colonies were counted.

A polyphasic taxonomic approach was used to identify bacteria isolated from the chemostats, which were characterized on the basis of their gram-staining characteristics, morphology, fermentation products, biochemical reactions in API 20A and 32A tests and analysis of cell membrane fatty acid methyl esters (FAME). Bacterial cellular fatty acids are stable and reproducible taxonomic markers, which allow comparisons of patterns of their methyl esters to be made by gas chromatography with the microbial identification system (MIDI). Cellular fatty acids were extracted from overnight cultures of the organisms grown in MIDI PYG broth. After centrifugation to obtain a cell pellet, FAME were produced by saponification, methylation, and finally solvent extraction. FAME were then separated with a 5898 A MIDI (Microbial ID, Inc., Newark, Del.). They were automatically integrated, and numerical analysis was done with standard MIS library generation software.

Short-term fermentation studies on biofilm and planktonic populations. Culture (200 ml) was taken from both chemostats. The biofilm that formed on the wall of vessel 1 was also removed at this time. After washing and resuspension in 100 mM sodium phosphate buffer (pH 6.0), the samples were centrifuged at 13,000 × *g* (20 min). The resulting pellets were each resuspended in 20 ml of anaerobic phosphate buffer (0.4 M; pH 6.0). Ten milliliters of each suspension was added to 40 ml of chemostat medium in 70-ml serum bottles (Jencons, Forest Row, East Sussex, United Kingdom) under N_2 at 37°C. Samples were taken periodically for up to 6 h and centrifuged at 13,000 × *g* (10 min). The cell-free supernatants were then frozen at −20°C for subsequent measurement of SCFAs. Samples were also frozen for analysis of residual mucin. Dry-weight determinations were made of the samples for calculations of specific rates of substrate uptake and utilization.

Short-chain fatty acid analysis. SCFAs were first acidified with 50 mM H_2SO_4 to convert them into free fatty acids, which were subsequently extracted into ether (25). SCFA were separated by gas chromatography with an Agilent Technologies model 6890N gas chromatograph, following split injection (40:1), using a HP-INNOwax (cross-linked polyethylene glycol) column, 30 m by 0.25 mm by 0.25 μm (no. 19091N-133; Agilent Technologies). Injector and detector temperatures were 250°C and 300°C, respectively. The initial column temperature (120°C) was held for 1 min and increased thereafter in stages of 10°C per min until 265°C was reached and maintained for 2 min. Flow rates of the helium carrier gas, H_2, air, and N_2 (makeup gas) were set at 1.8 ml min^{-1}, 40 ml min^{-1}, 450 ml min^{-1}, and 45 ml min^{-1}. All samples were quantitated by comparison of sample peak heights with those of authentic standards and internal standards with Hewlett Packard Integrated Chemstation software.

Electron microscopy. Mucin gels were examined by scanning electron microscopy. Samples from the gel surface were washed first with potassium phosphate buffer (0.1 M; pH 7.0) before being placed in 3% (vol/vol) glutaraldehyde in PIPES buffer [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid; 0.1 M and pH 7.4] at room temperature for 1 h and then stored at 4°C. The samples were subsequently fixed with 4% (wt/vol) aqueous OsO_4 and dehydrated stepwise in ethanol, which involved three changes (10 min each) in 50%, 75%, 95%, and finally 100% ethanol. They were then dried on a Poleron E5000 critical-point dryer and placed on stubs before being gold coated to a depth of 30 nm and viewed with a Philips XL 30 FEG electron microscope.

Oligonucleotide probes. The fluorescent 16S rRNA oligonucleotide probes in this study have been previously described. Probe Bif 164 (5′-CATCCGGCATT ACCACCC-3′) was used to identify bifidobacteria (22), Ent (5′-CCGCTTGCT CTCGCGAG-3′) was used for enterobacteria (24), and Bac 303 (5′-CCAATG TGGGGGACCTT-3′) was used for bacteroides and prevotella (41). Intestinal isolates and a range of culture collection type strains were used as controls in testing specificities of the oligonucleotide probes (12). Organisms were cultured in Wilkins-Chalgren broth in an anaerobic chamber (atmosphere: N_2, 80%; CO_2, 10%; H_2, 10%) at 37°C. The bacteria were then fixed in fresh paraformaldehyde, washed in phosphate-buffered saline (PBS), and stored at −20°C (2). Hybridization at 50°C without formamide was found to give optimum conditions for the simultaneous visualization of the three probes in the mucin gels. Probes were synthesized by Thermohybaid, Interactiva Division (Ulm, Germany) and 5′ labeled with the fluorochromes Cy3, fluorescein isothiocyanate (FITC), or Cy5.

Fluorescent in situ hybridization (FISH). Gels were rapidly frozen in Tissue-Tek freezing medium (Sakura Finetek Europe B.V., The Netherlands), cut into 10-μm-thick sections with a cryomicrotome, and placed on Teflon-coated 10-well glass slides (VWR; Merck Eurolab, Ltd., Poole, United Kingdom). After being air dried, they were fixed in fresh 4% paraformaldehyde in PBS (pH 7.0) for 1 h at room temperature and washed with PBS (10 min). After being air dried, each well had 40 μl of hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl, 0.01% sodium dodecyl sulfate, pH 8.0) added, containing either FITC or Cy3- or Cy5-labeled probes at concentrations of 30 to 50 ng/μl. Hybridization was done at 50°C in a humid chamber in a hybridization oven for 2 h. Unbound probe was subsequently removed with 2 ml wash buffer (0.9 M NaCl, 20 mM Tris-HCl), and the slides were placed in 50 ml of wash buffer for 20 min at 50°C. Hybridized slides were rinsed with distilled H_2O and air dried. Citifluor (Citifluor, Ltd., London, United Kingdom) was used as a mounting medium, and the slides were visualized with a Nikon Eclipse E800 upright microscope attached to a Nikon PCM 2000 confocal system. Bacteria labeled with FITC probes were detected with a 488-nm argon laser (green), bacteria labeled with Cy3 probes were detected with a 543-nm helium-neon laser (red), and bacteria labeled with Cy5 probes were detected with a 650-nm helium-neon laser (blue). Images were captured and overlaid with C-Imaging software (Compix, Inc., Cranberry Township, PA).

Carbohydrate measurements. Neutral and amino sugars in mucin were separated by high-pressure anion exchange chromatography with pulsed amperometric detection on a Dionex Carbopac PA 10 (4 by 250 mm) column equipped with a Dionex PA 10 guard column (4 by 50 mm) and a Dionex ED 40 detector with a Dionex DX 500 system, according to the method of Quigley and Englyst (48). The sugars were hydrolyzed in 2 M H_2SO_4 for 2 h at 100°C. A standard mixture of each sugar (1.0 mg/ml) was also treated with 2 M H_2SO_4. Five milliliters of internal standard solution (0.02 mg/ml deoxygalactose in high-purity water) was added to 0.1 ml of hydrolysate or sugar standards and mixed. Isocratic separation of fucose, galactosamine, glucosamine, and galactose was achieved at 1.0 ml/min with 40 mM NaOH. Mannose was isocratically separated at 1.0 ml/min with 4 mM NaOH. A Dionex Peaknet software data handling system was used to plot and integrate the results. *N*-Acetyl neuraminic acid was determined by hydrolyzing samples in 0.05 M H_2SO_4 for 1 h at 80°C. Released neuraminic acid was determined colorimetrically by the thiobarbituric acid method (49, 56) using a Philips PU 8740 UV/VIS scanning spectrophotometer at 549 nm.

Rates of mucin carbohydrate uptake in the chemostats were calculated as follows: $q_s = D\,(S_o - S)/x$, where D = dilution rate, S_o = substrate entering fermentor, S = residual substrate in fermentor, and x = community dry weight (q_s = substrate utilized per minute per milligram [dry weight] of bacteria). In mucin gels, q_s was calculated as $S_o - S/t$, where S_o = initial substrate concentration, S = substrate remaining, and t = time.

Glycosidase assays. Glycosidase activities were assayed by the addition of 0.5 ml of sample to 0.25 ml of various *p*-nitrophenyl substrates and incubation at 37°C until activity could be detected by the development of a yellow color, as *p*-nitrophenol was released, or for 1 h if no visible yellowing was evident. The reaction was then stopped by the addition of 0.75 ml 500 mM Na_2CO_3–$NaHCO_3$ solution, and the absorbance was measured at 420 nm (28). The following substrates were dissolved in Tris-HCl buffer (10 mM, pH 6.5) to give 15 mM solutions: *N*-acetyl-α-D-galactosaminide, α-L-fucopyranoside, *N*-acetyl-β-D-glucosaminide, and β-D-galactopyranoside.

In the neuraminidase assays, the test solution (0.05 ml) and boiled controls were incubated with 0.01 ml *N*-acetylneuraminlactose (1 mg/ml) in acetate buffer (100 mM, pH 5.5) for up to 2 h at 37°C. The reactions were stopped by being boiled at 100°C for 2 min. Released neuraminic acid was determined as described above.

Culture dry weights. These were determined by methods described by Degnan and Macfarlane (11).

Chemicals. Bacteriological culture media and supplements were supplied by Oxoid, Ltd. (Basingstoke, Hampshire, United Kingdom). Unless otherwise stated, all chemicals and reagents were obtained from Sigma Chemical Co. (Poole, Dorset, United Kingdom).

TABLE 1. Comparison of predominant planktonic populations in the continuous culture system and those associated with wall growth in vessel 1[a]

Bacterial population	Vessel 1		Vessel 2 planktonic
	Planktonic	Wall growth	
Total facultative anaerobes	6.7 ± 0.3	8.3 ± 0	7.0 ± 0.3
Enterobacteria	6.7 ± 0.3	8.3 ± 0	7.0 ± 0.3
Facultative anaerobic cocci	5.0 ± 0.2	4.7 ± 0.1	5.3 ± 0.2
Total anaerobes	10.4 ± 0	9.9 ± 0.1	10.2 ± 0.1
Total bacteroides	10.1 ± 0	9.5 ± 0.1	10.2 ± 0.1
B. thetaiotaomicron	10.0 ± 0.2	9.2 ± 0	9.2 ± 0.3
B. uniformis	10.1 ± 0.1	9.2 ± 0.1	9.8 ± 0.1
B. caccae	ND[b]	ND	9.7 ± 0.1
Anaerobic gram-positive cocci	5.9 ± 0.3	ND	5.7 ± 0.2
Total bifidobacteria	7.5 ± 0.1	5.9 ± 0.1	7.0 ± 0.1
B. longum	7.4 ± 0.1	5.9 ± 0.1	7.0 ± 0.1
B. adolescentis	7.2 ± 0.1	ND	ND
B. pseudolongum	6.9 ± 0.2	ND	6.2 ± 0.1
Eubacteria	6.7 ± 0.3	6.2 ± 0.2	6.5 ± 0.2
Fusobacteria	ND	ND	ND
Clostridia	8.5 ± 0	6.5 ± 0.1	8.7 ± 0.1
C. clostridioforme	8.5 ± 0	6.5 ± 0.1	8.7 ± 0.1
C. perfringens	6.8 ± 0.2	ND	6.7 ± 0.1
C. malenominatum	7.1 ± 0.1	5.9 ± 0	6.6 ± 0.2
Lactobacilli	5.8 ± 0.3	ND	5.7 ± 0.2

[a] Results are mean $\log_{10}$ CFU/ml culture ± standard deviation ($n = 2$).
[b] ND, not detected.

RESULTS

Predominant planktonic and wall growth communities in the fermentors. Complex populations of intestinal bacteria were maintained in both chemostats throughout the experiment (Table 1), with anaerobes greatly outnumbering facultative anaerobes in the planktonic phase. The principal culturable anaerobes were identified as species belonging to the genera *Bacteroides*, *Clostridium*, and *Bifidobacterium*. Some species, such as *Bacteroides caccae*, only occurred in high numbers during growth under strictly carbon-limited conditions in vessel 2. Fusobacteria, lactobacilli, and anaerobic cocci were not detected or occurred in low numbers in the fermentors, while enterobacteria were the main facultative species. Counts of these organisms were 3 orders of magnitude lower than the bacteroides. With the possible exception of *B. thetaiotaomicron*, no major differences were evident in planktonic populations in vessels 1 and 2.

Significant chemostat wall growth formed rapidly in vessel 1, but macroscopic wall growth was never detected in the second fermentation vessel. Bacteriological analysis showed marked differences in the biofilm, with increased proportion of enterobacteria and reductions in the relative numbers of bifidobacteria, anaerobic gram-positive cocci, and clostridia. Thus, planktonic communities in vessel 1 and vessel 2 more closely resembled each other than the planktonic and biofilm populations in vessel 1.

Colonization of mucin gels by fecal bacteria. Mucin gels were placed in the fermentation vessels when the bacteria were growing under steady-state conditions to investigate invasion and destruction of the mucus substratum. Rapid colonization of mucin gels by fecal bacteria occurred in both culture vessels (Fig. 2). However, bifidobacteria grew slowly in the mucin and only under nutrient-limited conditions in vessel 2, where they achieved high cell numbers. In general, anaerobic bacteria in the mucin biofilms did not reach the cell population densities seen in the planktonic phase. While there was little difference in rates of bacteroides growth in mucin in the fermentors, *Bacteroides distasonis*, which was not detected in the planktonic phase, only colonized gels in vessel 1, while *B. caccae* growth was slow and was only apparent in mucin gels in vessel 2 (Fig. 3). It was also evident that mucin biofilm communities contained proportionally higher numbers of enterobacteria than the planktonic phase in the chemostats. This was a general characteristic of microbial biofilms in the fermentors, where planktonic anaerobe:facultative anaerobe ratios in vessel 1 were 5,000:1, whereas the corresponding chemostat wall population was 40:1. In the mucin gels, the ratios were 3:1 (vessel 1) and 6:1 (vessel 2). The electron micrographs in Fig. 4 show that after a 24-h immersion in the fermentors, dense bacterial populations of various morphologies had covered the mucin surface. FISH, with a mixture of three genus-specific 16S rRNA oligonucleotide probes (Fig. 5A), showed that the predominant biofilm species bacteroides and enterobacteria grew in close juxtaposition to each other in the mucus and that the bacteroides often formed microcolonies (Fig. 5B).

Mucin breakdown in planktonic and biofilm populations. The principal mucin sugars in the growth media were galactose, *N*-acetylglucosamine, *N*-acetylgalactosamine, and fucose, with lesser amounts of *N*-acetyl neuraminic acid and mannose (Table 2). The vast majority of carbohydrate was utilized by intestinal bacteria in fermentor 1, where mannose and fucose were completely fermented. However, residual oligosaccharide sugars, particularly galactose, were detected in culture effluent in fermentor 2. In the mucin gels, most of these carbohydrates were digested to some extent by fecal bacteria, apart from *N*-acetyl galactosamine in vessel 1. With the exception of mannose in this fermentor, none of the mucin oligosaccharide sugars were completely fermented. Destruction of mucin in the gels was most extensive by bacteria growing under strongly nutrient-limited conditions in vessel 2.

Specific rates of utilization of mucin oligosaccharide sugars by planktonic and biofilm populations are given in Table 3. The data show that mannose, galactose, and *N*-acetyl neuraminic acid were fermented most rapidly by the planktonic communities, whereas galactose and *N*-acetylglucosamine were utilized most effectively in the gels. Apart from mannose and from the *N*-acetyl neuraminic acid in vessel 2, specific rates of breakdown of mucin carbohydrate constituents were notably higher in the gels, particularly in fermentor 2.

Measurements of hydrolytic enzymes. Table 4 shows mucinolytic enzyme activities of bacteria growing in the mucin gels and their corresponding (steady-state) planktonic and vessel 1 wall biofilm activities in the fermentors. With the exception of *N*-acetyl neuraminidase, mucinolytic activities were generally

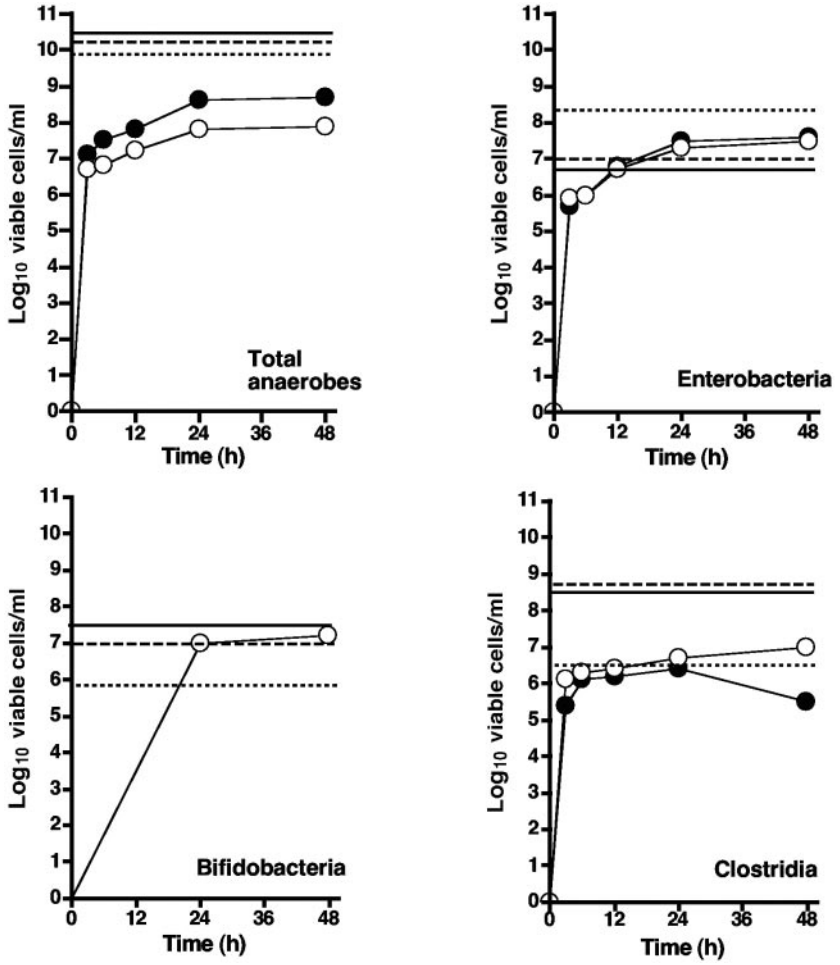

FIG. 2. Development of bacterial populations in mucin biofilms. Closed circles, vessel 1; open circles, vessel 2. The solid line shows steady-state planktonic populations in vessel 1, and large broken lines show those in vessel 2. Small broken lines indicate bacterial numbers in the chemostat wall biofilm in vessel 1.

lower than in the planktonic phase. Apart from *N*-acetyl neuraminidase, enzyme activities were comparable in the mucin gels in the two chemostats.

Fermentative activities of planktonic and wall populations in the chemostats. Results in Table 5 demonstrate that under steady-state conditions, acetate was the major SCFA in both fermentors, although the relative proportions of propionate and butyrate were higher in vessel 1.

Measurements of maximum specific rates of SCFA formation demonstrated that acetate and butyrate production by planktonic bacteria in fermentors 1 and 2 was more rapid than in microbial communities growing in the chemostat wall biofilm. Rates of propionate formation were, however, comparable. The molar ratios of SCFA produced by different bacterial populations in the chemostats were markedly different from their steady-state values. Thus, acetate formation by planktonic bacteria from vessel 1 was substantially reduced, with concomitant increases in propionate and, to a lesser degree, butyrate.

The chemostat wall populations from vessel 1, in contrast, mainly produced acetate and virtually no butyrate. Acetate was the principal fermentation product formed by planktonic organisms from vessel 2, but propionate and butyrate ratios were higher than the corresponding steady-state chemostat values.

DISCUSSION

The two-stage continuous fermentation system used in this investigation for studying the formation of mucin-degrading biofilms enabled colonization of the glycoprotein gels to be examined under nutrient and energy-rich conditions characteristic of the proximal bowel (vessel 1) and the same bacteria to be investigated under carbon and energy-limited conditions similar to those in the distal bowel (vessel 2) (40). Inoculation of the chemostat with fecal bacteria has been shown previously to effectively simulate colonic populations that occur in vivo (33). The growth medium contained a complex mixture of (growth-limiting) proteins, carbohydrates, and mucin to maximize species diversity in the fermentors. Partially purified porcine gastric mucin was used in the experiments because of its compositional and structural similarity to human colonic mucin (55).

The experiments were designed to investigate colonization of artificial mucus gels by colonic bacteria and to study the

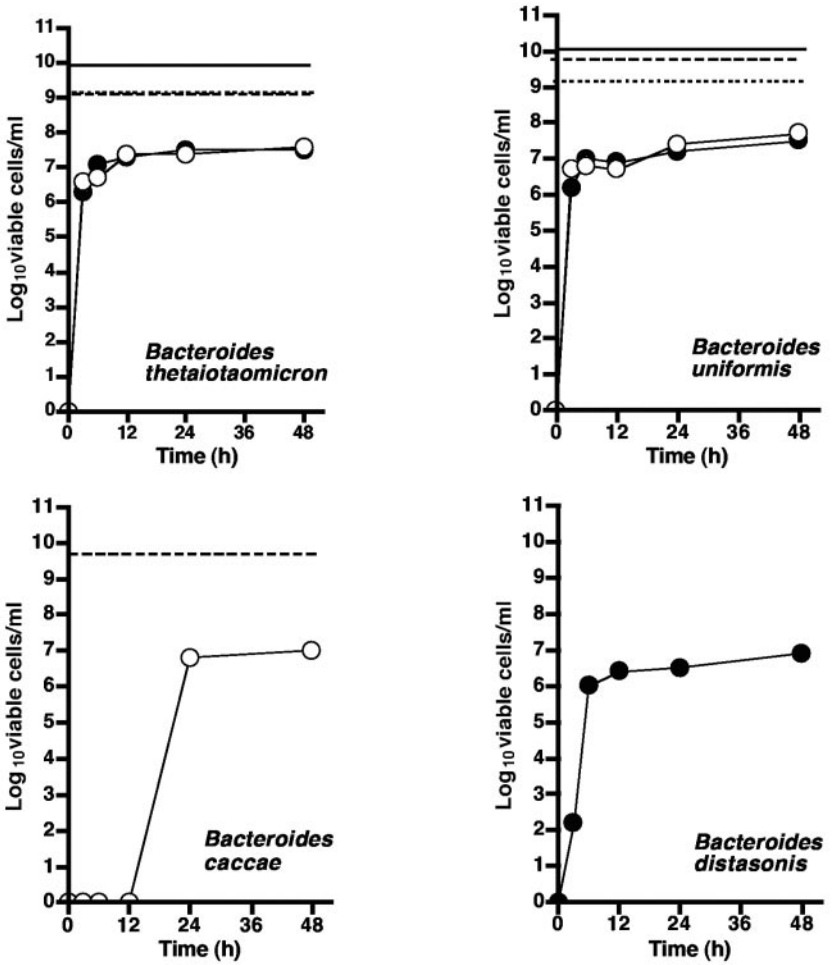

FIG. 3. Development of bacteroides populations in mucin biofilms. Closed circles, vessel 1; open circles, vessel 2. The solid line shows steady-state planktonic populations in vessel 1, and large broken lines show those in vessel 2. Small broken lines indicate bacterial numbers in the chemostat wall biofilm in vessel 1.

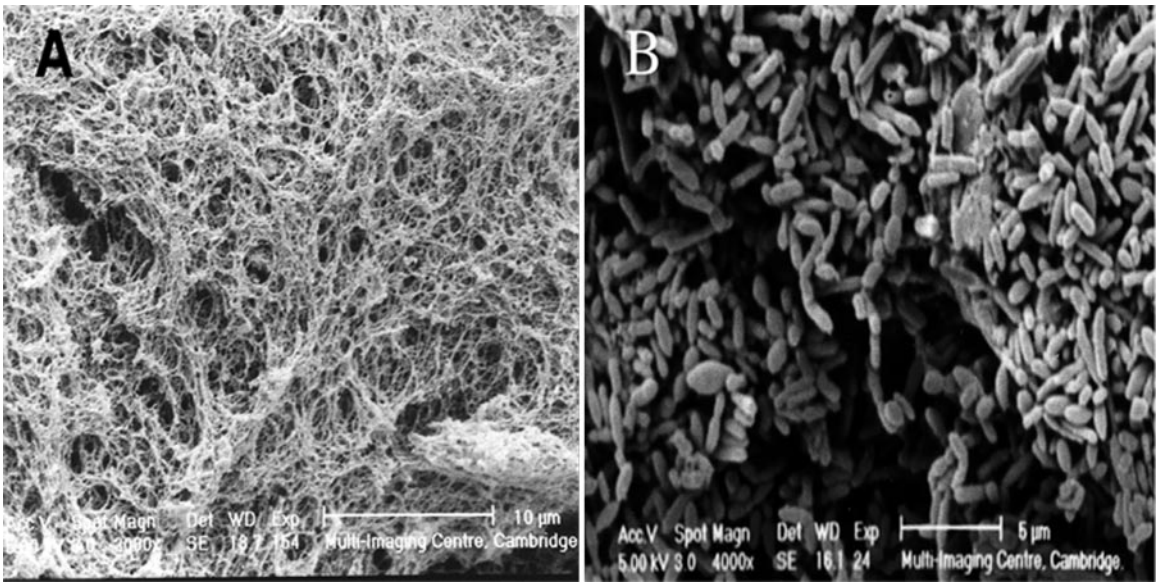

FIG. 4. Colonization of mucin gels in vessel 2 by fecal bacteria. (A) Scanning electron micrograph of mucin gel at zero time. (B) Scanning electron micrograph of mucin gel after 24-h immersion in the chemostat.

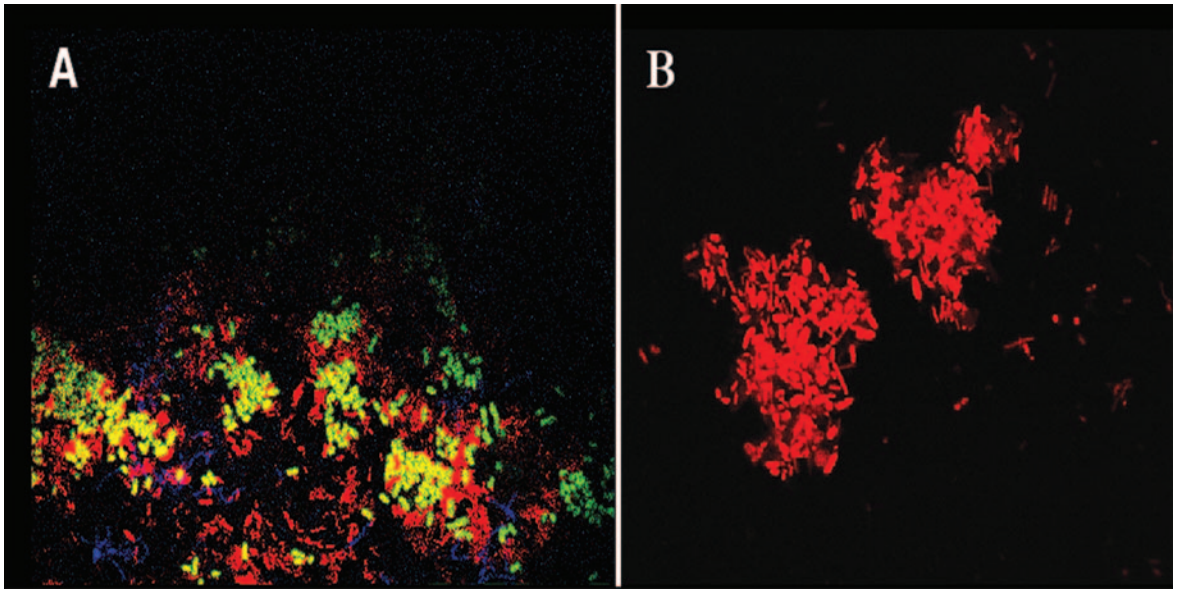

FIG. 5. Fluorescent light micrograph of mucin gels labeled with 16S rRNA oligonucleotide probes. (A) Mucin gel at 12 h, showing enterobacteria (FITC; green), bacteroides (Cy3; red), and bifidobacteria (Cy5; blue). (B) Bacteroides microcolonies at 12 h, labeled with a genus-specific oligonucleotide probe (Cy3). Magnification, ×1,000.

utilization of mucus oligosaccharides during biofilm formation. Other studies have reported that bacterial colonization of mucus is rapid, typically occurring within 15 min of contact with bacterial cells (14, 15); however, the rate and the way in which intestinal bacteria colonize the mucus layer are unknown. Salivary oral mucins play a protective role in the oral cavity by modulating colonization and facilitating removal of pathogens, and rapid attachment of multispecies planktonic aggregates has also been demonstrated to occur in vitro on saliva-coated coupons. The establishment of mucus-degrading microbial consortia occurred rapidly in this study, as shown by the early adherence and growth of members of the *Bacteroides fragilis* group, clostridia, and enterobacteria. A diverse range of mucin-degrading and nonmucinolytic bacteria from the large intestine rapidly established in the gels, with anaerobic organisms, particularly bacteroides, predominating. Bacteroides have also been reported to be the predominant species found on colonic and rectal epithelial surfaces in vivo (9, 35, 47) and are probably the principal polysaccharide-degrading bacteria in the gut, due to their ability to grow on a variety of polysaccharides and synthesize a wide range of cell-associated polysaccharide depolymerases and glycosidases, Some bacteroides could only colonize mucus under energy-limiting (*B. caccae*) or energy-excess (*B. distasonis*) conditions, indicating that diet can affect the growth of different types of bacteria in mucus. The significance of dietary influences on mucosal bacteriology has recently been shown with mouse models and with humans, where oligofructose feeding greatly increased mucosal bifidobacterial numbers (21, 23).

Culturing studies showed that bifidobacterial colonization was slow and only occurred during carbon-limited growth (Fig. 2). The predominant bacteria in the mucin biofilms were generally similar to those in the planktonic phase of the chemostats; in numerical terms, none of the microbial populations detected in the gels exceeded those in the planktonic phase in the fermentors, with the exception of the enterobacteria. These organisms were ubiquitous in the mucin biofilms, occurring in high numbers, which has also been observed to occur on the

TABLE 2. Mucin utilization by planktonic bacterial populations and mucin biofilm communities

	Carbohydrate concn (μg/ml)[a]				
	Planktonic phase			Mucin gel[b]	
	Feed medium	Vessel 1	Vessel 2	Vessel 1 initial	Vessel 2 initial
N-Acetyl neuraminic acid	80 ± 8	45 ± 1 (44)[c]	20 ± 2 (75)	140 ± 8 (71)[d]	135 ± 7 (82)
Fucose	95 ± 3	ND (100)	ND (NA)	132 ± 10 (46)	120 ± 10 (58)
N-Acetyl galactosamine	138 ± 10	22 ± 0 (84)	20 ± 1 (86)	205 ± 16 (7)	210 ± 18 (55)
N-Acetyl glucosamine	225 ± 15	52 ± 0 (77)	40 ± 2 (80)	390 ± 21 (46)	390 ± 26 (60)
Galactose	760 ± 20	195 ± 8 (74)	190 ± 11 (75)	640 ± 27 (31)	635 ± 32 (38)
Mannose	47 ± 3	ND (100)	ND (100)	122 ± 11 (100)	110 ± 7 (70)

[a] Results are means from two experiments ± standard deviation.
[b] Mucin carbohydrate concentrations at zero time.
[c] Values in parentheses show mean percent utilization of individual mucin sugars.
[d] Values in parentheses indicate percentage carbohydrate utilization after 48-h colonization of the mucin in the chemostats.

TABLE 3. Specific rates of mucin carbohydrate utilization (q_s) by planktonic and biofilm communities

	Sugar utilization (μg/h/mg [dry wt] of bacteria)			
	Vessel 1 planktonic	Vessel 2 planktonic	Vessel 1 mucin gel	Vessel 2 mucin gel
N-Acetyl neuraminic acid	1.9 ± 0.10	2.8 ± 0.09	2.5 ± 0.05	2.1 ± 0.04
Fucose	0.2 ± 0.05	0.3 ± 0.04	1.4 ± 0.18	1.8 ± 0.10
N-Acetyl galactosamine	0.9 ± 0.21	1.1 ± 0.16	0.8 ± 0.04	2.9 ± 0.19
N-Acetyl glucosamine	1.0 ± 0.15	1.2 ± 0.19	4.0 ± 0.27	5.3 ± 0.30
Galactose	3.2 ± 0.18	3.9 ± 0.24	4.5 ± 0.31	5.6 ± 0.36
Mannose	4.5 ± 0.21	5.2 ± 0.38	3.2 ± 0.19	2.4 ± 0.12

colonic and rectal mucosa (16, 17, 35, 45) showing that the model was an effective simulator of bacterial growth in mucin in vivo. In the gut lumen, facultative anaerobes are usually outnumbered by a factor of 100 to 1,000 by strictly anaerobic species (19). The increase in the facultative anaerobe:anaerobe ratio in both chemostat wall growth and mucin gels, compared to the planktonic populations in the chemostats, was found to be similar to values obtained from colonic tissue at autopsy (9) and rectal biopsies, where strict anaerobes outnumbered facultative species by a factor of 5 to 10 (35).

Scanning electron microscopy of gel slices (Fig. 4) showed a variety of bacterial morphologies in the mucin, while 16S rRNA FISH probes demonstrated a predilection for microcolony formation by the bacteroides, which has also been reported to occur on the large bowel mucosa (35, 37). Bifidobacteria were usually more widely dispersed in the mucin. The use of rRNA probes with different target specificities provided evidence of colocalization between bacteroides and enterobacteria on mucin gels, indicating possible metabolic dependence.

Mucin in culture medium supplied to the fermentors was extensively degraded by planktonic bacteria, especially in fermentor 1 (Table 2). The residual mucin carbohydrate found in the planktonic phase in vessel 2 was considered to constitute an indigestible fraction, in view of the nutrient-poor conditions, and the limited amount of oligosaccharide taken up by bacteria in this chemostat. Development of oligosaccharide-degrading enzyme activities occurred only after substantial bacterial populations had become established in the gels, indicating that they did not play a major role in the initial stages of mucin colonization (data not shown).

Determinations of residual carbohydrate in the mucin gels demonstrated that the majority of glycoprotein oligosaccharide was utilized by bacterial communities that formed in the gels, though at different rates. Interestingly, *N*-acetyl neuraminic acid was assimilated rapidly, even though initial neuraminidase activities were low in the mucin, while a marked lag was observed with galactose and fucose uptake and with *N*-acetyl galactosamine in vessel 2, despite the existence of significant glycosidase activities. These observations show that microbial catabolite regulatory mechanisms were affecting the way mucin oligosaccharides were being utilized (10, 27, 40).

While the mechanisms involved in oligosaccharide uptake in mucin gels are unclear, liberation of a diverse range of monosaccharides from mucin carbohydrate chains is considered to be an important contributary factor in maintaining species diversity in colonic and oral ecosystems (6, 20). In vitro studies with *Bacteroides fragilis* suggest that through catabolite regulation of metabolism, short-term accumulation of sugars during depolymerization of mucin oligosaccharide chains could also have important consequences with respect to the physiology and ecology of other species growing in mucus-degrading microenvironments (28). The fact that bacterial species composition and hydrolytic enzyme activities were generally similar in mucin gels in the two fermentation vessels suggests that rapid sugar uptake was probably due to higher transport activities in catabolite-regulated bacterial sugar transport systems in vessel 2.

Intestinal bacterial fermentations are regulated by the need to maintain redox balance, principally through the reduction and oxidation of ferredoxins, flavins, and pyridine nucleotides. To a large degree, this affects the flow of carbon through the bacteria, the energy yield obtained from the substrate, and the fermentation products that can be formed. Synthesis of reduced products including H_2, lactate, succinate, butyrate, and ethanol is used to effect redox balance during fermentation, whereas the formation of more oxidized substances such as acetate is associated with ATP generation. Conversely, more reduced fermentation products result in lower ATP and growth yields (29).

The abilities of gut bacteria growing in biofilms on the wall of the first-stage fermentor and planktonic populations in both culture vessels to ferment purified mucin and other polymer-

TABLE 4. Oligosaccharide-degrading activities in mucin biofilms and planktonic populations in chemostats

	Activity (U/ml)[a]				
	Vessel 1 planktonic	Vessel 1 wall growth	Vessel 2 planktonic	Vessel 1 mucin gel	Vessel 2 mucin gel
N-Acetyl neuraminidase	168 ± 7	243 ± 14	184 ± 6	1,025 ± 130	760 ± 50
α-Fucosidase	160 ± 1	58 ± 2	297 ± 2	61 ± 3	76 ± 2
N-Acetyl α-galactosaminidase	104 ± 2	101 ± 2	100 ± 0.1	45 ± 2	37 ± 1
N-Acetyl β-glucosaminidase	996 ± 9	673 ± 1	225 ± 0	320 ± 0	400 ± 25
α-Galactosidase	721 ± 28	241 ± 32	1,052 ± 33	185 ± 15	160 ± 23
β-Galactosidase	1,381 ± 38	641 ± 33	1,692 ± 26	530 ± 30	480 ± 20

[a] Data are means of results obtained in two experiments ± standard deviation.

TABLE 5. Comparative maximum rates of formation and molar ratios of SCFA produced by bacteria growing in the continuous culture system

Parameter studied	Location of sample	Acetate	Propionate	Butyrate
Steady-state molar ratios in chemostats	Vessel 1	70	17	13
	Vessel 2	83	9	8
Rates of SCFA production in short-term fermentation studies[a]	Vessel 1 (planktonic)	3.7 ± 0.1	4.5 ± 0.5	4.7 ± 0.1
	Vessel 1 (wall growth)	2.3 ± 0	4.9 ± 0.5	2.2 ± 0.1
	Vessel 2 (planktonic)	4.1 ± 0.2	5.0 ± 0.5	4.3 ± 0.2
SCFA molar ratios in short-term fermentation studies[b]	Vessel 1 (planktonic)	55	26	19
	Vessel 1 (wall growth)	78	21	1
	Vessel 2 (planktonic)	70	17	13

[a] Values are millimoles per hour per milligram (dry weight) of bacteria. Results are means of results obtained in two experiments ± standard deviations.
[b] Mean values after 2-h incubation.

ized carbon sources were investigated in this study to determine their metabolic capabilities. Bacterial communities in the fermentors were shown to be functionally distinctive with respect to their metabolic characteristics (Table 5). These experiments were done using anaerobic, nitrogen-limited, short-term batch culture fermentations to preclude growth and changes in bacterial populations during the course of the experiment (39). The results showed that although maximum specific rates of SCFA formation were greatest in the planktonic fractions (Table 5), acetate and propionate molar ratios associated with vessel 1 wall growth were very similar to their steady-state values in the fermentor. In contrast, considerably more butyrate was formed by nonadherent bacteria.

Bacterial mass measurements in the fermentors showed that the majority of cells were associated with wall growth in vessel 1 (4.4 g [dry weight]). Values for the planktonic phases were 2.6 g and 1.7 g for vessel 1 and vessel 2, respectively. It can be therefore be seen that biofilm bacteria growing on the chemostat wall made the principal contribution to acetate production, whereas nonadherent organisms were largely responsible for the majority of propionate and butyrate formation. The high molar ratios of acetate in the planktonic microbiota in the second-stage fermentor showed that the organisms were growing under extremely energy-limiting conditions. This was even more apparent in the biofilm communities in wall growth in the first-stage fermentation vessel, which is explicable in terms of mass transfer limitation effects involved in sequestration of substrate from the planktonic phase (50).

In conclusion, we know surprisingly little about the fine structure of bacterial mucin-degrading communities in human intestinal biofilms, their ecological significance in the colonic ecosystem as a whole, or their metabolic importance to the host. However, evidence suggests that these microbiotas are heterogeneous assemblages that form rapidly either on the surfaces of particulate matter in the intestinal lumen or in the mucous layer lining the mucosa (34, 39). While the in vitro modeling studies described here provide useful comparative information on the physiological activities of biofilm and nonadherent populations, the analytical methods employed were essentially destructive and did not provide information concerning the multicellular organization of the biofilm communities or of the spatial relationships between different groups of bacteria that play an important role in gut physiology.

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